Crystal structures of zinc-free and -bound heme domain of human inducible nitric oxide synthase

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INTRODUCTION

Nitric oxide synthases (NOS) are a family of enzymes that produce nitric oxide (NO) and L-citrulline from L-arginine in the presence of NADPH and O_2. NOS maintains a biodomain structure with the catalytic center residing in the heme domain utilizing electrons derived from the cytochrome P450 reductase-like biflavin domain. The heme domain also contains the binding site for the enzyme cofactor, tetrahydrobiopterin (H_4B). The NOS family currently consists of three mammalian isoforms. The endothelial (eNOS) and neuronal (nNOS) isoforms are constitutive and are activated by Ca^{2+} - dependent calmodulin binding. The inducible (iNOS) entertains a tightly bound calmodulin subunit and is regulated at the level of transcription.

The structure of the eNOS heme domain revealed a novel zinc tetrathiolate (ZnS_4) at the bottom of the dimer interface [1]. In the structure of the heme domain of murine iNOS [2], a disulfide was identified in the Zn binding site and no Zn was seen in the structure. To understand the structural consequences of the metal center in other NOS isoforms, we have solved the crystal structure of human iNOS in both Zn-free and -bound forms.

METHODS

The human iNOS heme domain (residues 1-504) was cloned and expressed in E. coli. The protein sample (residues 74-504) used for crystallization was generated by trypsinolysis upon the purified iNOS heme domain. Rod shaped crystals were obtained using sitting drops in a sandwich box setup at 7°C with a protein concentration of 10 mg/ml. The reservoir solution contained 30 mL of 35% saturated ammonium sulfate, 30% (v/v) glycerol, 50 mM sodium citrate, pH 5.0. The solution used for setting up protein drops is identical to the reservoir solution except that 2.0 mM glutathione (GSH) and 10 mM L-Arg (or 1.0 mM S-ethyl-isothiourea) were also present. The Zn-containing crystals were grown

with 5.0 mM $ZnS0_4$ and 2.5 mM tris-(2-carboxyethyl)phosphine added to replace GSH in the Zn-free condition. The same drop setup solution also served as the crystal storage and cryoprotectant reagent.

Crystals were flash cooled under a cold nitrogen stream (100 K) for data collection. The Zn-free data (3.0 Å) were collected using a Mar Research imaging plate on beamline 7-1 at SSRL and the Zn-bound data (2.55 Å) on a Quantum4 CCD detector on beamline 5.0.2 at ALS. Raw data were integrated and scaled with DENZO and SCALEPACK. Crystals were indexed in a primitive tetragonal space group $P4_32_12$ with cell dimensions a=b=187.25 Å, c=227.49 Å. The iNOS heme domain structure was solved by molecular replacement using the dimeric eNOS heme domain as the search model. The Zn-bound structure was refined using X-PLOR with final R = 0.209 and Rfree = 0.243.

RESULTS AND DISCUSSION

As in the murine iNOS structure [2], the human iNOS structure also reveals a disulfide bond formed between symmetry related Cys 115 residues at the dimer interface. In sharp contrast, eNOS has a Zn ion tetrahedrally coordinated to pairs of symmetry related Cys 96 and Cys 101 residue [1]. The Zn binding motif, Cys-$(X)_4$-Cys, conserved in all known NOS isoforms to date, suggests that the physiologically relevant NOS structure has the Zn ion bound. The eNOS heme domain, which retains the Zn, was prepared by proteolysis from full-length recombinant eNOS whereas both the murine and human iNOS heme domains were expressed as the heme domain alone. Thus, we postulate that the presence of the reductase domain in the generation of eNOS heme domain may have aided in retaining the bound Zn.

If the Zn site is the "natural" form, then it should be possible to reconstitute the Zn site in iNOS. This was accomplished by simply adding Zn^{2+} in the presence of a strong reducing agent directly to the crystallization cocktail. The topology of the metal binding motif is nearly identical to that found in bovine eNOS. The Zn is located along the dyad axis of symmetry and is tetrahedrally coordinated to pairs of symmetry-related Cys residues. The Zn is equidistant from both H_4B molecules (~12 Å) and both heme iron atoms (21.8 Å). The strategically located metal center underscores its important structural roles that include maintaining the integrity of the pterin-binding pocket and possibly providing a docking surface. The electrostatic potential in the vicinity of the ZnS_4 of iNOS is positive and

similar to that observed in eNOS [1] and could provide a docking site for the negatively charged reductase domain.

A comparison of the Zn-free and -bound iNOS structures also illustrates the interplay between the Zn center and the pterin binding site. In the Zn-free human iNOS heme domain structure the formation of a disulfide between symmetry-related Cys 115 residues causes the peptide flip of Gly 117. This change, in turn, affects H_4B binding by weakening the H-bond between >C=O of Ser 118 and the pterin dihydroxypropyl side chain, which stretches to 3.3 Å from 2.7 Å in the Zn-bound structure. Therefore, it is not too surprising that replacement of the Zn Cys ligands with other residues through site-directed mutagenesis leads to weakened pterin binding as reported in the literature, most likely due to changes in pterin-protein interactions similar to that found by comparing the iNOS Zn-free and -bound structures.

Another role for the ZnS_4 is in maintaining dimer stability. In going from the Zn-free to the -bound state, there is a net gain of eight H-bonds which would contribute favorably to the free energy of dimer stabilization. Even though the Zn-free iNOS heme domain dimer has an intermolecular disulfide bond, the key H-bonds required to stabilize the structural elements in this region are absent. This argues for the inability of a disulfide to substitute for the Zn at the NOS dimer interface. In addition, strongly reducing conditions of the cytosol do not favor disulfide formation, further supporting the view that NOS utilizes Zn binding to amplify the conformational stability of the dimer interface.

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